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ORIGINAL PAPER

Silencing of Gm*FAD3* gene by siRNA leads to low α-linolenic acids (18:3) of *fad3*-mutant phenotype in soybean [*Glycine max* (Merr.)]

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Abstract RNA interference (RNAi) has been recently employed as an effective experimental tool for both basic and applied biological studies in various organisms including plants. RNAi deploys small RNAs, mainly small interfering RNAs (siRNAs), to mediate the degradation of mRNA for regulating gene expression in plants. Here we report an efficient siRNA-mediated gene silencing of the omega-3 fatty acid desaturase (FAD3) gene family in a complex genome, the soybean (*Glycine max*). The FAD3 enzyme is responsible for the synthesis of α-linolenic acids (18:3) in the polyunsaturated fatty acid pathway. It is this fatty acid that contributes mostly to the instability of soybean and other seed oils. Therefore, a significant reduction of this fatty acid will increase the stability of the seed oil, enhancing the seed agronomical value. A conserved nucleotide sequence, 318-nt in length, common to the three gene family members was used as an inverted repeat for RNA interference. The RNAi expression cassette was driven by a seed-specific promoter. We show that the transgene-produced siRNA caused silencing of *FAD3* that was comparable to the *fad3* mutant phenotype and, furthermore, that such a silencing is stably inherited in engineered soybean lines. Since the pool size of the α-linolenic acids is small relative to the other polyunsaturated fatty acids in soybean, the significant reduction of this fatty acid suggests a role and great potential for the siRNA strategy in silencing gene families in a complex genome.

Keywords RNAi · siRNA · Inverted repeat · Gene silencing · Gm*FAD3*

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Introduction

Down-regulation of endogenous plant genes is an important strategy to determine gene functions and

for efficient metabolic engineering. In recent years, RNA interference (RNAi) has gained significant attention because of its success in gene silencing in various organisms, including plants (Watson et al. 2005). In plants, the RNAi pathway primarily deploys small interfering RNA (siRNA) to mediate sequence-specific degradation of target mRNA (Baulcombe 2004; Broderson and Voinnet 2006; Jones-Rhoades et al. 2006; Vaucheret 2006). More recently, RNAi-based techniques have been used to down-regulate metabolic pathways' genes to improve nutritional value in several different plant species (Tang and Galili 2004). For example, RNAi has been successfully employed in cotton (*Gossypium* spp.) to down-regulate two key fatty acid desaturase genes encoding stearoyl-acyl-carrier protein Δ9-desaturase and oleoyl-phosphatidylcholine ω6-desaturase (Liu et al. 2002). Knockdown of these two genes in cotton leads to nutritional improvements in high-oleic and high-stearic cottonseed oils, which are essential fatty acids for human health. In another study, RNAi was used in coffee (*Coffea arabica*) plants to suppress the caffeine synthase gene, thus reducing caffeine content (Ogita et al. 2003). It is now clear that RNAi technology represents an effective tool to investigate many biological processes in plants and will provide synergistic opportunities to advance plant biology studies.

Soybean (*Glycine max*) is one of the most important crops worldwide. This species owes its economical value to its high seed protein and oil contents. However, soybean is considered to be one of the most difficult crop species to study transgene regulation due to the size and complexity of its genome and to the lack of efficient genetic engineering processes (Shoemaker et al. 1996, 2006). The functional redundancy and complex feedback regulation, largely owing to over 90% duplicated genome regions and to a large number of gene families, have been an acute obstacle for efficient gene regulation through transgenic approaches in this species. Several significant improvements have recently been made in molecular genetic tools for soybean. Soybean has a large expressed sequence tag (EST) database derived from a diverse set of expression libraries and several forms of gene chips are now available that will allow more quantitative and global gene expression analyses. To complement these developments, several plant-based RNAi cloning vectors are commercially or publicly available. In addition, the latest advances in soybean transformation (Zeng et al. 2004) allow for a more thorough study of gene overexpression and RNAi-mediated gene silencing on a whole-plant basis. Because of its high efficiency, we explored RNAi as a down-regulation strategy to study gene regulation in the soybean. One additional impetus for our investigation stemmed from the lack of sufficient studies on siRNA-mediated gene silencing in stably-transformed soybean plants derived from *Agrobacterium*-mediated T-DNA transfer.

In this report, we chose the omega-3 fatty acid desaturase (FAD3) gene as a target to investigate RNAi efficacy in soybean. *FAD3* controls important seed traits and represents a gene family of three active members: Gm*FAD3A*, Gm*FAD3B*, and Gm*FAD3C*, which encode three corresponding microsomal forms of FAD3 enzymes (Ohlrogge and Browse 1995; Bilyeu et al. 2003). The enzymes catalyze the conversion of linoleic acids (18:2) to α-linolenic acids (18:3) in the polyunsaturated fatty acid (FA) biosynthesis pathway during seed development. These microsomal forms are primarily responsible for generating the α-linolenic acids (7–10% of the oil fraction) in the soybean seed. It is mostly this small, unstable FA that contributes to reduced soybean seed oil quality and the generation of undesirable "*trans*-fatty acid" (as a result of hydrogenization), despite the healthy benefit of this oil (Dutton et al. 1951; Kinney 1996; Bilyeu et al. 2006). Therefore, a significant reduction of α-linolenic acids will improve soybean oil quality; however, this reduction will not be possible unless all three active gene family members are simultaneously down-regulated (Bilyeu et al. 2006). Our working hypothesis in this study is that the use of a highly-conserved nucleotide sequence that represents a domain common to gene family members as an RNAi target site will permit silencing of all three gene family members. An additional advantage of assaying the function of the *FAD3* gene is the ease of FA analysis, which is achieved using gas chromatography (GC), a simple, fast, and high-throughput technique. Therefore, *FAD3* represents an excellent model to test the efficacy of RNAi-mediated silencing of gene families, which account for a large number of soybean genes. Additionally, to date there has been only very limited studies published on RNAi-mediated silencing of *FAD3* in plants; our

experiments should thus enhance understanding of this area.

Here we show that siRNA generated from a transgene containing inverted repeats (IR) representing a domain conserved among gene family members is effective in silencing *FAD3* in engineered soybean, leading to very low level of α-linolenic FA, a phenotype similar to that of an *fad*-mutant phenotype. We further demonstrate that this silencing is inheritable. Our results suggest a role and great potential for siRNA in silencing genes with multi-gene family members in a complex genome, a strategy that promises to be very valuable for trait improvement.

Materials and methods

Plant materials

Soybean genotype 'Jack' was obtained from Illinois Foundation Seeds (Champaign, IL) and used for development of transgenic soybean lines. All soybean plants, either wild type or transgenic lines, were grown in 13-liter pots containing Pro-mix soil and release-fertilizer Osmocot 14-14-14 (Hummert International, Earth City, USA) under greenhouse conditions. The greenhouse conditions included supplemental light with intensity 50–90 Klux and 12:12 h photoperiod from late May to early November and a 14:10 h photoperiod during the remaining seasons. The temperatures were set between 23 and 26°C (average: 24°C) throughout the seasons. Plants were fertilized once at the beginning of planting or at transplanting, and watered as needed.

RNAi vector construction

RNAi expression vectors of the pMU103 series were constructed on the basis of the binary plant transformation vector, pZY101.1 (Frame et al. 2002). pMU103.1 contains a 333-bp *Eco*R I-*Hin*d III fragment from vector pRTL2 (Carrington 1990) that includes the TEV and CaMV35S promoter. Vector pMU103.1 was further modified by subcloning a *Nco* I-*Hin*d III fragment from the vector pMCG161 that contains a cassette for hairpin RNA production. The resulting vector, pMU103.2, contains the rice waxy-a intron flanked by the polylinkers for cloning of inverted repeats and a transcriptional terminator. Finally, the soybean glycinin seed-specific promoter was cloned into the *Nco* I and *Asc* I sites to generate vector pMU103.

RNAi target design and amplification

The soybean *FAD3* EST clone (Genbank accession number BE210900) was procured from Biogenetic Services, Inc (Brookings, SD). A 318-bp conserved domain of the *FAD3* ORF was detected by a BLAST search of the entire Genbank database (Fig. 1a). This domain was then PCR-amplified to incorporate restriction sites for subsequent cloning into the pMU103 vector. The PCR primers are: forward, 5′-CTTAATTAAGGCGCGCCTGCTCAAGCTCTATGG-3′; reverse, 5′-GCGGATCCATTTAAATGAAATGGTAATGGTGCAG-3′. The PCR reaction conditions were: 2 min at 94°C for hot start, 30 s at 94°C for denaturing, 30 s at 65°C for annealing, 1 min at 72°C for extension, 30 cycles of run, and 5 min at 72°C for final extension. The resultant vector pMU-FAD (Fig. 1b), carrying the Gm*FAD3* inverted repeats (IRs), was used for soybean transformation experiments.

Plant transformation

Soybean transformation followed our latest protocol, which is an improved version derived our previously published methods (Zeng et al. 2004). The system utilizes *Agrobacterium tumefaciens*-mediated T-DNA transformation, the *bar* (*bi*alaphos *r*esistance) gene as a plant selectable marker coupled with glufosinate as a selective agent (Aventis CropScience, Research Triangle Park, NC). Major modifications include the addition of dithiothreitol (DTT) and sodium thiosulfate to the co-cultivation medium at a concentration of 1 mM each, in addition to the L-cysteine, which was used at a concentration of 3.3 mM (Olhoft et al. 2003). We also used a new selection scheme, namely: 0, 10, and 5 mg l^{-1} of glufosinate at the first and second shoot initiation and shoot elongation stages, respectively. T-DNA insertion was initially verified by an herbicide leaf-painting assay (Zhang et al. 1999). Independent transgenic soybean lines were initially verified by recovering them from

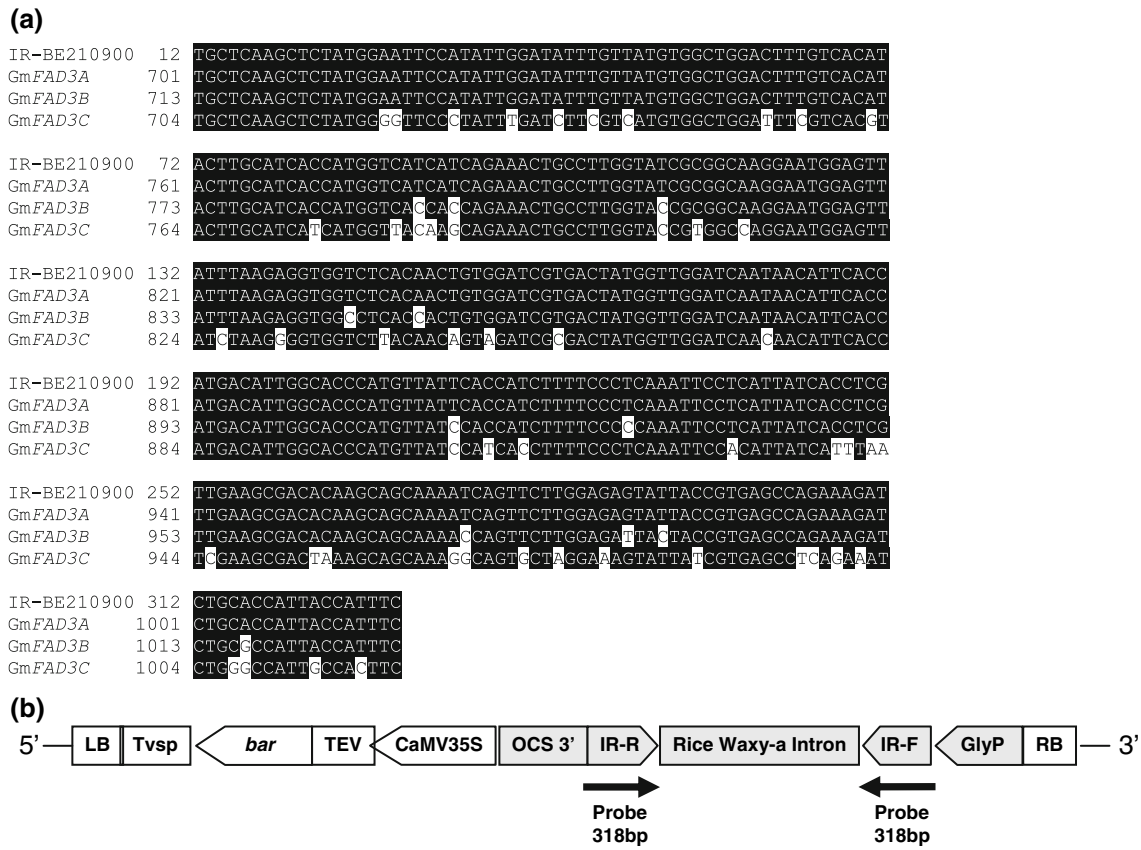

Fig. 1 Design and assembly of the expression cassette for the RNAi of Gm*FAD3*. (**a**): CLUSTAL-W nucleotide sequence alignment of the 318-nt region of soybean EST clone BE210900 (genotype "Ogden") with partial cDNAs of Gm*FAD3A*, Gm*FAD3B*, and Gm*FAD3C* (genotype "Jack"). Conserved nucleotides are shown in boxes. The position of each *FAD3* sequence is marked beginning from start codon; whereas the position of the EST clone is marked from the first nucleotide of the EST sequence. (**b**): Schematic presentation of the T-DNA region of the plant transformation vector, pMUFAD. LB and RB, T-DNA left and right borders, respectively; Tvsp, soybean vegetative storage protein gene terminator; *bar*, *b*ial*a*phos *r*esistance gene; TEV, tobacco etch virus translational enhancer; CaMV35S, tobacco mosaic virus 35S promoter; OCS 3′, octopine synthase gene terminator; IR-R and IR-F, the 318-bp inverted repeats of Gm*FAD3* target sequence in reverse and forward orientations, respectively; Rice Waxy-a Intron, rice *Waxy*-A gene intron; Gy1P, soybean glycinin gene promoter

different explants and later confirmed by progeny and phenotypic analysis. Herbicide resistant soybean lines and their progeny were subsequently subjected to molecular and phenotypic analysis.

Identification of RNAi homozygous lines

Progeny plants (T_1) from parental lines (T_0) hemizygous for RNAi transgenes were screened for herbicide resistance using leaf-painting (Zhang et al. 1999). T_2 plants derived from individual T_1 RNAi lines were screened again by leaf-painting assays. Those T_1 lines whose progeny plants (over 100 plants per line) all displayed herbicide resistance were considered to be RNAi homozygous lines and therefore were used for further molecular analysis.

Amplification of full-length GmFAD3A, GmFAD3B, and GmFAD3C cDNAs

Since partial Gm*FAD3* cDNA sequences from soybean genotype Jack were available from the GenBank database, 5′RACE and 3′RACE analyses were performed to obtain the full-length 5′ and 3′ ends of all three FAD3 transcripts (GeneRacer kit, Invitrogen). A two-step PCR strategy was employed: for an initial

amplification, a common primer to the region of high conservation in all *FAD3* sequences was used along with the GenRacer primer. The 1st PCR reaction was used as a template for a subsequent amplification with a gene-specific primers and corresponding GenRacer nested primers. Reverse primers for the 5′RACE: *FAD3* general, 5′-GGCTAATTCTCCATCCATGG-TATGG-3′; *FAD3A*, 5′-ACTGGGATCAAAAGCTT CCTTTTGG-3′; *FAD3B*, 5′-GCTAGGATCAAAAT CAAAAGAAGAACCC-3′; *FAD3C*, 5′-GTTGTAGA GGCTGTGCTTGAACCATTTT-3′. Forward primers for the 3′RACE: *FAD3* general, 5′-CATTCACCATG ACATTGGCACCCATG-3′; *FAD3A*, 5′-CCGAGAC TGAGTTTCAATTTTTGGGTTATTT-3′; *FAD3B*, 5′-ACGAGACTGAGTTTCAAACTTTTTGGGTTA TTA-3′; *FAD3C*, 5′-CAACTAAAGTTTTTGATGC-TACATTTACC-3′. The RACE PCR products were amplified with the MasterTaq DNA polymerase (Eppendorf) cloned into the pCR4-TOPO vector (Invitrogen) and then sequenced. For final sequencing, long 5′RACE PCR was performed with gene-specific primers complementary to those for the 3′RACE. PCR products of ~1.2 kbp were amplified with proof-reading *Pfu Turbo* DNA polymerase and then cloned and sequenced. The full-length cDNA sequences were deposited into Genbank with assigned accession numbers: Gm*FAD3A* (EF632324), Gm*FAD3B* (EF632325), and Gm*FAD3C* (EF632326).

Genomic DNA analysis

Southern analysis was used to confirm different integration events. Genomic DNA of soybean lines homozygous for the RNAi transgene was extracted from leaf tissues as previously described (Dellaporta et al. 1983) with a few modifications. Twenty μg of DNA were digested with a restriction enzyme (either *Hin*d III or *Xho* I) that cut once within the T-DNA region. The digested DNA was fractionated in a 1.0% agarose gel and transferred to a Zeta-Probe® GT nylon membrane (Bio-Rad, CA). DNA was fixed to the membrane by UV cross-linking. Hybridization and washing conditions followed the protocols of Southern (1975) and the Zeta-Probe® GT manufacturer's instructions. The 318-bp DNA fragment by itself corresponding to the *FAD3* gene target for RNAi was used to generate a ^{32}P-labeled probe (Prime-it® II kit, Stratagene).

mRNA analysis

Total RNA was isolated from 0.1 g of mid-mature seeds using the Trizol reagent. Ten μg of total RNA from each plant sample, along with the RNA ladder, were fractionated on a 1.0% agarose gel, transferred onto a Zeta-Probe® GT nylon membrane (Bio-Rad), and fixed by UV cross-linking. Conditions for hybridization and washing of membranes were as described previously (Zhang et al. 1998). The conserved 318-nt DNA fragment itself was used to generate a probe. To generate the probes specific to Gm*FAD3A* and Gm*FAD3C* family members, 5′ UTR fragments of these genes' cDNAs were synthesized as sense strand and then used as templates with the anti-sense specific primers. Hybridization conditions for the gene-specific probes followed those described for small RNA analysis, except that α-^{32}P-dCTP was used.

Small RNA analysis

For detection of small (21-nt) RNA fragments, the procedure described by Hamilton and Baulcombe (1999) was used with a few modifications. To enrich for small RNA, total RNA was extracted from mid-mature seed samples using Trizol reagent (Invitrogen, Rockville, MD). Low molecular weight RNA was subsequently isolated by resuspending 100–200 μg of total RNA in 350 μl of diethylpyrocarbonate (DEPC)-treated water. Then 100 μl of 25% DEPC-treated polyethylene glycol (MW 8000) and sodium chloride (to a final concentration of 5%) were added and the solution was incubated on ice for 30 min. After removing the precipitate by centrifugation at 13,000 RPM at 4°C, the small RNAs remaining in the supernatant were transferred to a new tube and extracted with an equal volume of phenol/chloroform. The solution was then centrifuged for 10 min at 13,000 RPM at 4°C. The supernatant containing small RNA was removed, mixed with three volumes of EtOH, and placed at −20°C for at least 2 h before being centrifuged at 13,000 RPM for 10 min at 4°C.

The pellet was washed with 75% DEPC-treated EtOH and resuspended in 20 µl of DEPC-treated water.

A total of 30–50 µg of small RNAs, along with two types of sizing ladders, were resolved by electrophoresis on a denaturing 15% polyacrylamide gel (30:0.8) containing 7 M urea in TBE buffer (45 mM Tris-borate, pH 8.0, and 1.0 mM EDTA). The molecular weight ladders used for size estimation of small RNAs were synthetic single-stranded DNA oligos of 15-, 20-, 30-, and 50-nt length. These ladder sequences were derived from the *FAD3* target region. Blot hybridization analysis was performed as previously described (Llave et al. 2002) with some modifications. Briefly, small RNA was electroblotted to Hybond membranes (Amersham Biosciences, Pittsburgh, PA) using a trans-blot semidry-transfer cell (Bio-Rad, Hercules, CA) for 4–5 h at 30 V and then UV cross-linked. Radio-labeled probes for detecting small RNA were made as described for the Southern blot. Blot membranes used for the analysis of small RNA were prehybridized and hybridized at 40°C using PerfectHyb Plus buffer (Sigma, St. Louis, MO) for 2 h and overnight, respectively and washed at 50°C.

FA analysis and statistics

FA composition of dry mature soybean seeds from transgenic and wild type control samples were determined by a gas chromatography (GC) method (Cahoon et al. 2000). Four seeds were randomly chosen from each transgenic soybean line and wild type control for GC phenotypic analysis. However, only data from those seeds representing transgenic lines among segregating seed populations were used to calculate means. Data were analyzed for variance analysis using the SAS GLM program (Der and Everitt 2001), and means were separated by Duncan's Multiple Range Test at $\alpha = 0.01$ level.

Results

Potent silencing of Gm*FAD3* in engineered soybean

To effectively silence the three active members of the soybean *FAD3* gene family, we first identified highly conserved regions of the plant *FAD3*-encoded proteins. A BLAST search of the entire Genbank database using all available soybean *FAD3* ESTs as queries identified one highly conserved region that was specific only to the FAD3 protein among 17 (out of 19) plant species searched. This region includes the catalytic domain of the FAD3 protein owing to the presence of a high content of His residues (data not shown). One soybean EST clone (accession BE210900) showed the highest number of hits and the longest conserved region among the sequences analyzed. This region also shared a high degree homology with a 318-nt conserved region of three partial *FAD3* cDNA sequences, the only *FAD3* sequences from soybean genotype "Jack" available at that time: 100%, 96.5%, and 84.3% with Gm*FAD3A*, Gm*FAD3B*, and Gm*FAD3C*, respectively (Fig. 1a). These sequences are identical to those of three full-length *FAD3* cDNAs that we have more recently cloned from soybean genotype "Jack" (Genbank accession numbers EF632324, EF632325, and EF632326).

The sequence identity of clone BE210900 with the Gm*FAD3A* was thought to be essential for the efficient silencing, because this gene family member is largely responsible for seed α-linolenic acid synthesis (Bilyeu et al. 2003). Therefore, the 318-bp conserved region from the clone (BE210900) was chosen to generate the inverted repeat (IR); the sequence was PCR-amplified and then cloned into the RNAi cassette of the vector pMU103, which also carries the *bar* gene as a plant selectable marker (Fig 1b). To minimize the metabolic workload and potential risk of the "off-target" effects (Jackson et al. 2003) by RNAi of Gm*FAD3*, we used the soybean glycinin gene promoter (Cho et al. 1989; Sims and Goldberg 1989) to drive expression of the RNAi cassette. The glycinin gene promoter is seed-specific and shows highest expression at mid-seed maturity. The IR was separated by a functional intron from the rice waxy-a gene (Fig. 1b) to enhance the potency and stable inheritance of the RNAi (Smith et al. 2000).

Twenty-three independent primary (T_0) transgenic soybean lines were developed from genotype Jack with the construct pMUFAD that also carries the *bar* gene as a plant selectable marker (Fig 1b). All T_0 RNAi plants were first verified using an herbicide leaf-painting assay (Zhang et al. 1999); 14 of them

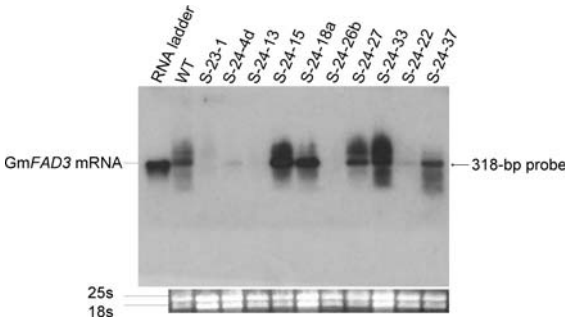

Fig. 2 Northern blot analysis of mid-mature seeds of primary *FAD3* RNAi lines. Total RNA was extracted from a random seed sample of each T_0 RNAi hemizygous line. Shown is the hybridization with 318-bp *FAD3* conserved probe. Bottom panel: ethidium bromide staining of the RNA gel (loading control). The RNA ladder is 1,500-nt in size. Note that each seed sample here was from a progeny population segregating for the transgene

were then analyzed by progeny segregation analysis. For a preliminary evaluation of silencing status, we randomly chose 10 RNAi lines and performed Northern analysis of total mRNA samples from one mid-mature seed (T_1) for each line (Fig. 2, top panel). As shown, in 5 out of 10 lines, i.e., S-23-1, S-24-4d, S-24-13, S-24-26b, and S-24-22, *FAD3* transcripts were not detected when probed with a 318-bp sequence conserved among all *FAD3* family members. Because transcripts of all three *FAD3* family members (*A*, *B*, and *C*) are about the same length and because no other band was detected, this result indicates that all three active *FAD3* genes were effectively silenced in the analyzed RNAi lines.

When T_1 mature seeds from all 23 RNAi lines were analyzed for the FA profile, 11 RNAi lines displayed a significant reduction in α-linolenic acid (18:3) content, ranging from 1.0% to 3.1% (Table 1). This low level of 18:3 FA phenocopies the soybean Gm*FAD3* mutant phenotype (Fehr et al. 1992; Rahman et al. 1998; Bilyeu et al. 2006), suggesting a potent silencing of Gm*FAD3*. Additionally, the linoleic acid (18:2) contents of all 11 RNAi lines were significantly increased relative to the wild type control (Table 1). This effect was apparently due to a block in the conversion of 18:2 to 18:3 FAs.

RNAi of Gm*FAD3* is inheritable

Next we tested if the high degree RNAi of Gm*FAD3* was inheritable and whether transgene zygosity or

Table 1 Fatty acid (FA) analysis of T_0 soybean for RNAi transgene

Line name	Fatty acid content (%)				
	16:0	18:0	18:1	18:2	18:3
WT	10.2	4.3	23.8	55.5a	7.1a
S-23-15A	9.7	5.1	14.8	66.0bc	1.2b
S-23-16	9.3	4.5	17.8	67.0bc	1.3b
S-23-18A	10.3	4.4	14.6	69.4bc	1.3b
S-24-1	9.9	5.3	15.0	68.5bc	1.3b
S-24-4D	9.6	5.2	14.5	65.4b	1.4b
S-24-11B	10.3	3.9	16.4	68.2bc	1.3b
S-24-13	9.6	3.6	19.6	65.2b	2.1bc
S-24-15	10.1	4.2	16.3	65.4b	2.3bc
S-24-16	9.1	3.9	18.6	65.3b	3.1bc
S-24-19C	10.8	3.9	14.5	65.8b	1.2b
S-24-32	10.3	3.2	19.8	65.7b	1.0b

Note: Only 11 lines displaying RNAi are listed. The content of each type of FA is expressed as a percentage of total FA using gas chromatography (GC). Data for FA analysis were from 2 to 4 replications (seeds) for each soybean line. All data were statistically analyzed, but mean comparisons were made only for 18:2 and 18:3 data. Means within the same column followed by the same letter were not significantly different from each other at $\alpha = 0.01$ level as detected by Duncan's Multiple Range Test. WT: wild type control

dosage affected RNAi efficiency in transgenic hemizygous and homozygous lines. We randomly selected 14 of the 23 lines for progeny segregation analysis using an herbicide leaf-painting assay. Thirteen lines exhibited either a 3:1 or 1:1 (R:S) segregation ratio for herbicide resistance (Table 2), which indicated the presence of single transgene locus (i.e. a simple insertion event) in each of these RNAi lines. The 1:1 (R:S) ratio was a result of chimerism, typical of transformants derived from direct shoot organogenesis protocol (Zhang et al. 1999; Zhanyuan Zhang, unpublished). One line fit a 15:1 (R:S) ratio, indicating two transgene loci. We further identified T_1 lines homozygous for the RNAi of Gm*FAD3* through progeny segregation analysis of T_2 plants. Like T_1 seeds, randomly selected T_3 seeds from 4 homozygous and 4 hemizygous Gm*FAD3* RNAi lines (T_2) displayed very low levels of α-linolenic acids (Table 3). There was no significant difference in the levels of seed α-linolenic acids between homozygous and hemizygous stages of the same lines or between T_1 and T_2 hemizygous lines. Moreover, the linoleic acid (18:2) content in all tested T_2 RNAi lines once

Table 2 Progeny segregation analysis of T_0 RNAi soybean lines

Lines	Total progeny	Progeny		Segregation: R:S ratio	χ^2-value	*P*-value
		R	S			
S-23-5	17	12	5	3:1	0.18	0.674
S-23-6A	18	8	10	1:1	0.22	0.637
S-23-13	18	11	7	1:1	0.89	0.346
S-23-14	18	14	4	3:1	0.07	0.785
S-24-4D	6	3	3	1:1	0.00	1.000
S-24-12	17	11	6	3:1	0.96	0.327
S-24-13	18	11	7	1:1	0.89	0.346
S-24-14	17	10	7	1:1	0.53	0.467
S-24-15	15	8	7	1:1	0.07	0.796
S-24-18A	14	12	2	15:1	1.54	0.214
S-24-26B	18	8	4	3:1	0.22	0.637
S-24-27	15	11	4	3:1	0.02	0.881
S-24-32	10	7	10	1:1	0.13	0.715
S-24-34	18	11	7	1:1	0.89	0.346

Note: Progeny R and S columns indicate the number of progeny plants showing herbicide resistant and susceptible, respectively. Resistant progeny plants were scored as R and susceptible ones as S. χ^2-test was used to test Goodness of Fit of observed progeny plants against theoretical number of plants showing R and S, respectively. A single degree of freedom was used to obtain *P*-values

Table 3 Fatty acid (FA) analysis of T_2 soybean for RNAi transgene

Line name	Fatty acid content (%)					Seed yield
	16:0	18:0	18:1	18:2	18:3	
WT	10.2	3.4	16.6	58.9a	10.6a	315
Homozygous						
S-24-4D	10.9	3.7	16.1	68.1b	1.2c	423
S-24-13	9.9	3.7	18.2	65.8b	2.4c	417
S-24-15	10.2	3.6	15.3	67.4b	3.6b	269
S-24-32	10.9	3.8	14.8	69.1b	1.3c	389
Hemizygous						
S-24-4D	10.7	3.8	14.2	68.2b	1.6c	684
S-24-13	10.1	3.7	15.9	68.5b	1.8c	628
S-24-15	10.4	3.1	16.3	66.9b	3.3b	445
S-24-32	10.2	3.6	14.8	61.8b	1.0c	640

Note: FA profiles of mature seeds from 4 homozygous, independent transgenic soybean lines and their corresponding hemizygous lines were analyzed against wild type control Jack. The content of each type of FA is expressed as a percentage of total FA using gas chromatography (GC). Data for FA analysis and seed yield were from 2 to 4 replications (seeds) for each soybean line. All data were statistically analyzed, but mean comparisons were made only for 18:2 and 18:3 data. Means within the same column followed by the same letter were not significantly different from each other at $\alpha = 0.01$ level as detected by Duncan's Multiple Range Test

again increased irrespective of homozygous or hemizygous status, owing to the block in the conversion of 18:2 to 18:3 FAs. Together, these results suggested that RNAi-directed silencing of Gm*FAD3* was stably inherited and unaffected by transgene zygosity or dosage.

There was neither obvious germination penalty and other abnormal developmental effects (data not shown) nor seed yield loss (seed weight and numbers) in transgenic RNAi lines displaying the *fad3* phenotype as compared with the wild-type control (Table 3). This is presumably because the seed-specific promoter of soybean glycinin gene, which shows the highest expression at seed mid-mature stage, was used to drive the RNAi expression.

Silencing of Gm*FAD3* corroborates the accumulation of siRNA in RNAi transgenic lines

To further characterize the transgenic RNAi plants, we advanced generations of four randomly-chosen independent transgenic lines with down-regulated 18:3 FA. First, we confirmed four independent RNAi lines homozygous for the Gm*FAD3* transgene, i.e., S-24-4d, S-24-13, S-24-15, and S-24-32, by Southern blot analysis (Southern 1975) (Fig. 3a). The variable band sizes above 6.5 kb indicate different loci of the integrated transgenes. Evidence that the soybean genome carries at least three endogenous *FAD3* loci was confirmed because there were two distinct hybridizing bands, with one band about 2–3 times the intensity of the other, in all five samples including the wild-type control.

We subsequently analyzed small RNA samples of mid-mature seeds (T_3) from each of the 4 RNAi homozygous lines. Small RNA hybridization results demonstrated that all 4 lines displayed small interfering RNA (siRNA) (Fig. 3b), suggesting a role for the siRNA pathway in the 18:3 FA down-regulation. Additionally, three of the four lines had high levels of siRNAs. These results show that in these RNAi transgenic lines the drastically decreased level of 18:3 FA corresponded to siRNA accumulation,

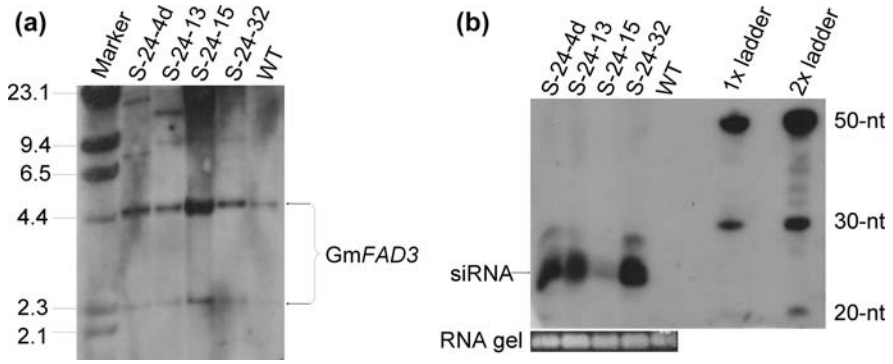

Fig. 3 Southern blot and small RNA analyses of *FAD3* RNAi homozygous lines. (**a**): Southern hybridization was performed with the 318-bp *FAD3* conserved probe. A *λ*/*Hin*d III ladder was used as molecular markers. (**b**): Small RNA detection among 4 RNAi homozygous lines using total RNA of T_3 seeds and 318-bp *FAD3* probe. Bottom panel: RNA gel showing loading control. Two marker lanes from left to right are 1× and 2× ladders using 3 and 6 ng synthetic DNA ladders, respectively, all of which are custom synthesized. RNAi homozygous lines used here were identified through herbicide-based leaf-painting progeny segregation analysis (see Materials and methods)

suggesting that siRNA of the transgene inverted repeats mediated the silencing.

Discussion

By employing a siRNA-producing transgene, we have silenced three active members of the *FAD3* family in soybean and achieved a high degree of down-regulation of α-linolenic acid, which mirrored the multiple Gm*FAD3* mutant phenotypes. This high level of silencing can be attributed to the targeting of a highly conserved domain of the FAD3 coding region. This domain is present in all three active microsomal members of the GmFAD3 family. The down-regulation of these genes improves the stability of the soybean oil and therefore minimizes the need for hydrogenization that results in the formation of *trans*-fatty acid. We further demonstrated that the potent silencing of Gm*FAD3* was inheritable and that it was not affected by transgene zygosity or dosage. Since the pool size (7–10% of total fatty acids) of α-linolenic acid is small relative to other polyunsaturated fatty acids, such silencing is in fact highly efficient. In addition, we believe that the high quality integration with simple insertion events and stable inheritance of transgenes, typical of T-DNA transfer, contributed to the high degree of gene silencing in the RNAi lines.

The ultimate goal in RNAi-mediated knock-down of a biochemical pathway or part thereof is the generation of strong, consistent and heritable events with desired trait modification(s). While the results we have described above are exciting and immediately promising, at this juncture it is important to discuss potential aspects of this approach that might be optimized in future experiments. For example, we did detect some variation in the down-regulated levels of 18:3 FA between different RNAi events or lines (Tables 1 and 3). Such variation could be caused by position-effect of the transgene as a result of differing chromosomal contexts of individual integration events that lead to varying levels of transgene expression (Dean et al. 1988; Peach and Velten 1991; Matzke and Matzke 1993). In addition, we detected residual levels of α-linolenic acid in our RNAi lines (as was the case in Gm*FAD3* mutant lines). This effect could simply result from different temporal expression patterns of the siRNA transgene and the endogenous Gm*FAD3* genes. Seed storage genes generally turn on later in seed development than do the fatty acid desaturase genes (Ruuska et al. 2002). Therefore it is possible that the siRNA transgene driven by the storage gene glycinin promoter is expressed later during the soybean seed development than is the *FAD3*; thus the *FAD3* temporarily escapes silencing. Yet, the use of such a seed specific promoter is still attractive because it did not cause a germination penalty, abnormal developmental effects or seed yield loss in our transgenic RNAi lines. Clearly, there is some room for optimization of parameters in the RNAi approach,

which we have demonstrated to be potentially very powerful strategy.

Previously, an antisense approach (Sivaraman et al. 2004) or both antisense and co-suppression strategy (Jadhav et al. 2005) were employed to silence *FAD2* gene family members in *Brassica*. Such silencing indirectly led to a reduction of 18:3 fatty acids to levels as low as 8–9.1%. Earlier and similarly, Kinney and Knowlton (1998) employed co-suppression strategy to silence the soybean *FAD3* gene, leading to a reduction of 18:3 to a level of 5%. Yet, each of these published studies clearly differs fundamentally from the RNAi-mediated silencing strategy that we employed in this study and neither Sivaraman et al. (2004) nor Jadhav et al. (2005) targeted the *FAD3* directly. Above all, none of these studies demonstrated such effective silencing of *FAD3* as we achieve here. More recently, Hirai et al. (2007) developed an in vivo assay based on the RNAi-mediated silencing of α-linolenic acid content in tobacco hairy roots. However, these authors demonstrated only a low degree of silencing, and their approach was based on a transient system. Finally, we must note that our approach seems to be similar to the RNAi-mediated silencing of a soybean *FAD3* gene, patented very recently (U.S. patent application 20060206963). While the patent document does not provide sufficient information for the public to evaluate their data, it does appear to corroborate and therefore validate our promising results.

Two additional RNAi studies in soybean are worth discussing: Subramanian et al. (2005) used RNAi technology to silence two copies of the soybean isoflavone synthase gene (IFS) to enhance susceptibility to *Phytophthora sojae*. However, these authors engineered soybean roots and cotyledons, not stably-transformed soybean plants, as we did. Nunes et al. (2006) studied RNAi-mediated silencing of the myo-inositol-1-phosphate synthase gene (Gm*MIPS1*) in transgenic soybean obtained by the biolistic technique. They demonstrated that silencing of the single Gm*MIPS1* gene inhibited seed development and reduced phytate content. Therefore, our results represent the first successful efforts employing RNAi both to silence a gene family in stably-engineered soybean plants derived from the T-DNA transfer process and to control the fatty acid profile of soybeans. Therefore, to the best of our knowledge, our study is the first public report employing RNAi to silence *FAD3* directly in soybean.

Our RNAi lines exhibited a *fad3* null-like phenotype, with extremely low levels of α-linolenic acids that corroborate the accumulation of siRNAs. The siRNA we detected is more likely 21-nt RNA because it mediates AGO-1 catalyzed sequence-specific cleavage of mRNA, as has been demonstrated in *Arabidopsis* and other plants. Our results here suggest a role of the siRNA in the silencing of *FAD3*. However, further experiments will be needed to determine the origin of the siRNA as to whether they are transgene-derived, degraded products of target *FAD3* transcripts, or both.

It will therefore be very interesting to further investigate whether siRNA can mediate potent and inheritable silencing of many other genes with multi-gene family members in complex genome as soybean. The information obtained from this study and future RNAi studies shall enhance metabolic engineering of many pathways and trait improvements in such economical important crops as soybean.

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